The Water-content in Artificially-ovulated Eggs of *Rana longicrus* and *Bufo melanostictus*

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There are many papers reporting the use of the pituitary in inducing ovulation in the following groups of vertebrates: fishes, snakes, doves, rats, cats, guinea-pigs and monkeys. As regards amphibia, there are papers by wolf (1929), Houssay, Giusti, Lascano-Gonza'ez (1929), Oshima (1937), besides that by Rugh (1935) who made some very fine experiments on *Rana pipiens*. According to their reports, there is little doubt that a suitable quantity of the pituitary extract by subcutaneous or peritoneal injection will cause ovulation.

However, there remain unsolved the following questions: What is the mechanics of ovulation after injection of the pituitary gland? What is the action of the pituitary hormone? How can one induce eggs to leave the ovary to constitute ovulation?

There has been no satisfactory explanation in answer to these questions. According to Samartino and Rugh (1945) the action of the pituitary gland is indirect. Ovulation can occur only in co-operation with the nervous and circulatory systems. The presence of that hormone alone could not induce ovulation. That is say, if immature ovary cultured in a solution of the hormone only will not show a least trace of maturation outside the female body. If a female with an isolated ovary in her body cavity is injected with the pituitary, no result will be got. If the communication with nervous and circulatory system is tied with threads and then injected with the pituitary, no result was obtained. It was found (Sarmatino and Rugh) that some substances such as colchicine or sodium fluoride, etc., mixed with the pituitary juice had no action on the ovary. These experiments support their original view that "the ovary of *Rana pipiens* did not respond to the anterior pituitary hormone in vitro."

But this was contradicted by the experiments of Heilbrunn, Daugherty and Wilbur, 1939; Grant 1940; Ryan and Grant 1940; McPhil and Wilbur 1943. Dr. Tchou-Su came to the conclusion that frog's eggs had to absorb a large quantity of water to enable

them to increase in size before they could leave the ovary. He argued that this increase in size caused an increase of internal pressure, which when it reached a certain point, burst the ovarian membrane to constitute ovulation.

With the above facts in mind, I was encouraged to make the following experiments under the supervision of Dr. Tchou. In the first place an attempt was made to determine whether ovulation in *Rana longicrus*, a common species in Taiwan, could be induced by the pituitary extract; secondly, if ovulation occurs, to estimate the water content in artificially ovulated eggs.

This experiment was carried out under the supervision of Dr. Tchou-Su, to whom I express my sincere thanks. Through the kindness of Dr. S. C. Hsu, Director of Taiwan Agricultural Research Institute, the present paper is published.

MATERIALS AND METHODS

Frogs belonging to the species *Rana longicrus* Stejneger, a common frog in Taiwan, were used for the experiment. They were gathered from the Botanical Garden inside our campus, Fountainhead of Taipeh city; Ts'ao-shan, etc. The experiments were carried out in October, a few months eariler than the natural breeding season which occurs from December to March of the next year. The pituitary extract came from the same species of frogs. It was found that the pituitary extract from the same species of animals was more potent than that from any other.

The frog was killed by pithing. The mucous membrane of the palate was cut away and the exposed brain case was removed by a pair of scissors. The pituitary--a pinkish organ of the size of a pinhead--was cut off. The pituitary glands from several specimens were ground in 0.5 cc. of distilled water or Ringer solution. The resulting whitish extract was then ready for injection. Subcutaneous injection was always preferred to avoid possible injury to the internal organs of the specimens in peritoneal injections.

After injection the specimens were kept in a glass bowl, containing water about 1 inch deep at a room temperature of about 20 °C.

OBSERVATION

(I) The effect of dosage:

The following table summarizes the effect of varying the quantity of injected material.

Table I.

Experiment No.	Number of Pituitary glands	Anatomical observation after 47—48 hrs.
1	3	No ovulation; oviducts markedly expanded.
2	3	No ovulation; oviducts markedly expanded.
3	3	No ovulation; oviducts markedly expanded.
4	3	No ovulation; oviducts markedly expanded.
5	3	No ovulation; oviducts markedly expanded.
6	5	No ovulation; oviducts markedly expanded.
7	5	No ovulation; oviducts markedly expanded.
8	5	No ovulation; oviducts markedly expanded.
9	5	No ovulation; oviducts markedly expanded.
10	5	No ovulation; oviducts markedly expanded.
11	8	No ovulation; oviducts markedly expanded.
12	8	Ovulation. A part of ovarian membrane ruptured. many eggs in the body cavity.
13	8	Ovulation. Good many eggs in the body cavity oviducts expanded.
14	8	No ovulation, oviducts expanded.
15	8	Ovulation. Complete rupture of ovarian membrane, good many eggs in the body cavity, four eggs already in oviduct.
16	10	Ovulation. Complete rupture of ovarian membrane, good many eggs in the body cavity, three eggs already in oviduct.
17	10	Ovulation. Complete rupture of ovarian membrane, good many eggs in the body cavity, thirteen eggs already in oviduct.
18	10	Ovulation. Eggs all in the oviduct and uterus. Set aside for artificial fertilization and good results obtained.
19	10	Ovulation. Partial rupture of ovarian membrane, four eggs in the body cavity.
20	10	Ovulation. All eggs in the uterus. Set aside for artificial fertilization and artificial parthenogenesis and good results obtained. Oviduct remarkably small.

4-5 glands sufficed to induce ovulation according to Rugh, using American frogs (*Rana pipiens* & *R. palustris*) and Tchou-Su & Chen Chao Hsi, using Shanghai frogs (*R. nigromaculata* & *Bufo bufo asiaticus*).

In this case 10 glands was needed. The first group of experiments were performed from Oct. 1st to Oct. 24th, 1947. From these experiments it may be concluded that the pituitary was effective in inducing ovulation in common Taiwanese frogs, although a large number of glands for every injection was necessary. There are exceptions where the ovary has not yet appeared black and the eggs are too young.

One inexplicable thing in this experiment is that injection of gonadotrophic extracts induces ovulation but never oviposition. Eggs were observed to be shed into the

body cavity before passing into and down the oviduct. They then descended en masse into the uterus where they were retained. Oviposition did not occur at all.

Dr. Tchou-Su suggested that oviposition might require some stimulus. So about 10 hours after injection, a normal male (not injected, because his nuptial pads is already much developed and is able to run after a female) was let in. Amplexus occurred, the eggs were shed. It was found that:

1. If a male was let in late, well fertilized eggs could not be obtained. (that is, only unfertilized eggs with a spotted surface signifying eggs had aged.)

2. If a male was in too early his advances would not be accepted by the female. But in one or two days, she was glad to receive him. During this time, some females were dissected. It was that the ovarian membrane was broken and the eggs had begun to ovulate. Sometimes the oviducts were full of eggs. In some cases eggs had already descended into the uterus. Hence it was desirable that the male be let in to the female immediately after iniection. Because we are convinced that male clasping will accelerate her oviposition.

3. The degree of descent of the eggs could be surmised by the extent of enlargement of the abdomen in the female after injection. Eggs in the uterus are good material for artificial fertilization, hybridation and artificial parthenogenesis.

(II) Increase of water-content during maturation in eggs of *Rana longicrus*.

Female of about the same size and freshly caught from the fields were each treated with ten pituitary glands. After 48 hours, the females were examined and their state of ovulation determined. The ovaries of these females are divided into 3 groups according to their weights. They were then kept in an oven. In the lst group, eggs had not ovulated, eggs had begun to ovulate but few were shed into the coelom in the second. In the last group the whole mass of eggs had ovulated, a part was retained in the coelom, the rest had entered the oviduct or even the uterus.

The total number of ovaries was twenty-one. Oviducts were similarly divided into 3 groups according to their water contents. The method of estimation of the water content is to weigh the eggs on a slide, and then put in an oven. At first they were weighed every other day. Temperature was gradually raised to about 60 °C till constant weights were obtained. Then the temperature was raised to 70°C at this temperature the weights remaind constant.

The results of the weighings of eggs and oviducts are tabulated in table II. In table III are weighings of ovaries of normal frogs untreated. This constitutes the control group. Table IV shows water-content of normal ovaries of frogs about to ovulate naturally.

TABLE II. Water-content of artificially-ovulated eggs and that of oviducts of frogs under the pituitary treatment.

Expt. No.	Wet Weight of egg mass gm.	Evaporated water wt. gm.	Percentage of evaporated water wt.	Wet wt. of oviducts gm.	Evaporated water wt. gm.	Percentage of evaporated water wt.
Before ovulation.						
30	2.5000	1.2950	51.80	0.8300	0.5500	66.27
31	1.1500	0.6200	53.91	0.4000	0.2900	72.50
32	1.7900	0.9700	54.19	0.4300	0.3400	79.07
34	1.8250	1.0800	59.18	0.8490	0.5700	64.14
35	1.6890	1.0050	59.50	1.0000	0.7500	75.00
36	2.7905	1.6100	57.70	1.4500	0.9500	65.51
Total	11.7445	6.5800		4.9590	3.4500	
Average	1.9574	1.0967	56.02	0.8265	0.5700	69.57
During ovulation.						
37	1.8000	1.0674	59.30	1.0060	0.7700	76.54
38	1.7880	1.0780	60.29	0.9500	0.6500	68.42
39	1.2500	0.7537	60.30	0.7810	0.5670	72.60
40	1.3600	0.8296	61.00	0.7000	0.5040	72.00
41	1.4000	0.8526	60.90	0.8500	0.6000	70.59
45	1.3000	0.7904	60.80	0.7500	0.5625	75.00
42	1.5000	0.9180	61.20	0.8000	0.5900	73.75
43	1.5300	0.9486	62.00	0.8300	0.6300	75.90
33	2.4000	1.4904	62.10	1.4000	0.8500	60.74
46	1.9000	1.1837	62.30	0.9000	0.7200	80.00
47	2.1000	1.3440	64.00	1.1000	0.7700	70.00
51	1.3000	0.8463	65.10	0.9000	0.7200	80.00
44	1.0100	0.6039	59.79	0.8000	0.6000	75.00
Total	20.6380	12.7066		11.7670	8.5355	
Average	1.5875	0.9774	61.57	0.9052	0.6564	72.51
After ovulation						
49	1.9000	1.2350	65.00	0.7000	0.5310	74.30
50	1.3000	0.8439	64.92	1.0900	0.8720	80.00
Total	3.2000	2.0789		1.7900	1.4030	
Average	1.6000	1.0395	64.96	0.8950	0.7015	78.38

TABLE III. Water-content of normal ovaries and oviducts of frogs in nature before ovulation.

Expt. No.	Wet Weight of egg mass gm.	Evaporated water wt. gm.	Percentage of evaporated water wt.	Wet wt. of oviducts gm.	Evaporated water wt. gm.	Percentage of evaporated water wt.
100	1.4500	0.7300	50.34	0.4200	0.3100	73.80
101	1.4000	0.7196	51.40	0.4000	0.2900	72.50
102	0.9000	0.4527	50.30	0.2500	0.1800	72.00
103	1.3500	0.7020	52.00	1.0100	0.7500	74.26

104	1.2300	0.6654	54.00	0.9000	0.6030	67.00
105	1.7900	0.9308	52.00	0.7000	0.4340	62.00
106	0.6100	0.3200	52.45	0.2000	0.1500	75.00
107	2.0080	1.0900	54.28	1.4000	1.0100	71.50
108	2.0000	1.0200	51.00	1.2000	0.8500	71.66
109	0.8000	0.4000	50.00	0.3000	0.2000	66.67
110	1.4300	0.7400	51.74	0.4000	0.2610	65.25
111	1.8000	0.9036	50.20	0.9500	0.6800	71.58
112	1.5000	0.7600	50.67	0.5000	0.3620	72.40
113	1.4900	0.7700	51.67	0.4300	0.3100	72.09
114	1.8000	0.9681	53.80	0.8500	0.5950	70.00
115	1.4000	0.7252	51.80	0.7600	0.5000	72.39
116	0.7000	0.3521	50.30	0.3200	0.2100	65.63
Total	23.6580	12.2498		10.9900	7.7050	
Average	1.3916	0.7206	51.78	0.6465	0.4532	70.10

TABLE IV. Water-content of eggs and oviducts during the period of maturation in nature.

120	1.3600	0.7602	55.90	0.9900	0.7350	74.24
125	1.9500	1.0920	56.00	1.2000	0.8400	70.00
130	2.2000	1.2716	57.80	1.4500	0.9800	67.58
132	1.8500	1.1118	60.10	1.1500	0.8510	74.00
Total	7.3600	4.2356		4.7900	3.4060	
Average	1.8400	1.0589	57.55	1.1975	0.8515	71.36

It may be noted from the above three tables that:

1. The water-content of the ovarirn eggs of *Rana longicrus* was 56.02%.
2. The water-content of eggs during maturation (at the period of ovulation) was 61.57%.
3. The water-content of mature eggs ready for fertilization was 64.96%.
4. This differences of the water content were found to exist in nature, but to a less extent. An average water-content of 51.78% compared with 57.55% of mature eggs.

From these weighings it may be concluded that artificially or naturally ovulated frog eggs gradually increased their water-content during the breeding season. Mature eggs may have 5.55% more water than the immature. Eggs ready for fertilization may have 3.39% more water than those that begin to mature.

(III) Increase of water-content during maturation in eggs of *Bufo melanostictus*.

A similar estimation of water-content was carried out in the case of *Bufo melanostictus* Only four pituitary glands from the same species was found sufficient to induce ovulation. The experiments were carried out in February near the natural breeding season. The temperature went as high as 21°C. Ovulation and oviposition occurred within 12 hours after pituitary treatment. The results are tabulated in Tables V and VI.

TABLE V. Water-content of articially ovulated eggs of *Bufo melanostictus*.

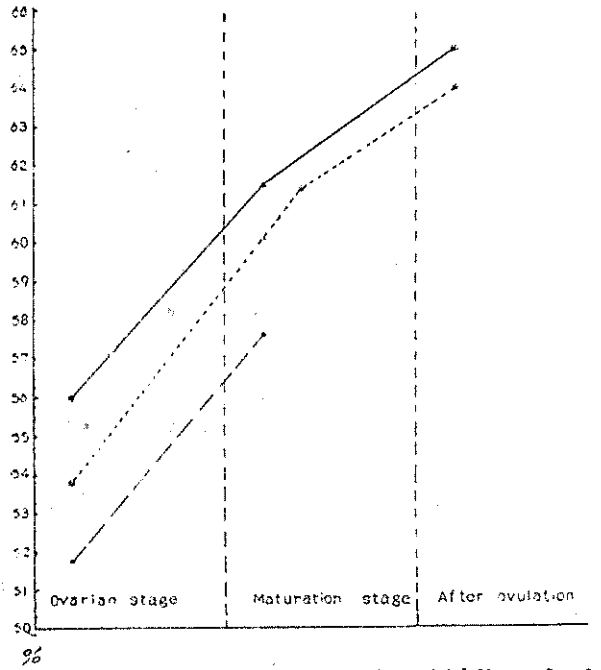
Expt. No.	Wet wt. of ovaries gm.	Evaporated water wt. gm.	Percentage of evaporated water wt.
Treated ovaries before ovulation			
8	9.2000	4.8760	53.00
11	8.4000	4.5384	54.10
12	14.0000	7.5950	54.25
14	9.3000	5.0034	53.80
16	7.2000	3.8880	54.00
Total	48.1000	25.9008	
Average	9.6200	5.1802	53.84
During ovulation			
a	15.3700	9.6200	62.51
b	17.5600	10.2700	58.49
c	7.8480	5.2480	66.80
d	9.7500	6.3000	65.64
e	14.3200	9.2293	64.45
f	12.4500	8.2344	66.22
g	15.2100	9.9626	65.50
h	9.3300	6.1765	66.20
i	18.3850	10.3100	56.00
j	19.7450	10.5840	53.61
Total	139,9680	85.9348	
Average	13.9968	8.5935	61.40
After ovulation.			
7	10.2500	6.5703	64.10
9	12.5000	8.0000	64.00
10	13.0000	8.3070	63.90
13	18.0000	11.5560	64.20
15	7.5000	4.7850	63.80
Total	61.2500	39.2183	
Average	12.2500	7.8437	64.00

TABLE VI. Water-content of ovaries of treated *Bufo melanostictus*.

Expt. No.	Wet wt. of ovaries gm.	Evaporated water wt. gm.	Percentage of evaporated water wt.
20	9.5300	5.1700	54.25
21	7.0200	3.7750	53.77
22	13.2220	7.1398	54.00
23	15.7400	8.4209	53.80
24	10.3000	5.5218	53.61
25	13.5100	7,3224	54.20
26	14.2000	7.7106	54.30
27	15.6100	8.4138	53.90
28	9.4800	5.1202	54.01
29	13.200	7.1333	54.04
Total	121.8120	65.7278	
Average	12.1812	6.5728	53.95

The conclusion to be drawn from these tables are 1. Mature eggs averaged 7.56% more water than the immature, 2. Eggs ready for fertilization averaged 2.63 % more water than the maturing eggs. The new paragraph increase of water content was shown for comparisen in the following figure I.

Figure 1: Increase of water-content of treated and untreated eggs.



Notes: ——— Percentage of evaporated water in artificially-ovulated eggs of *R. longicrus*.
..... Percentage of evaporated water in artificially-ovulated eggs of *B. melanostictus*.
------ Increase of the water-content of the ovaries of *R. longicurs* in nature.

DISCUSSION AND CONCLUSION

Reproduction is a big riddles.—In the present time though the science of biology has much advanced, yet the processes of reproduction are only partly assessed. In higher animals with bisexual method of reproduction, egg-laying may be taken as the onset of reproduction. (Growth of eggs is excluded because this is little known). Scientists in the past only knew that mature eggs would break out of the ovarian membranes to costitute ovulation at the breeding season. After delaying a short time in the coelom, eggs then enter the upper part of the oviducts; then descent to the uterus. In viviparous animals fertilized eggs remain here until they attain a certain stage of development. In oviparous animals, eggs are shed from the genital pore. It is a common rule with birds, mammals, and amphibians.

Scientists of late after careful investigations know that ovulation does has

something to do with the anterior lobe of the pituitary and that a female after treatment with the pituitary extract can be induced to ovulate. The result of this experiment is superficially satisfactory in that one can understand the truth of egg-laying, and control the action of ovulation. In short, there is little doubt that it is the action of the hormone in the pituitary glands.

How can the hormone induce the eggs to leave the ovaries and come out of the parent body to commence development? Is the influence of the hormone direct or indirect?

The experiment of Samartino & Rugh (1945) prove that this hormone did not affect the ovary directly but only in the presence of the nervous and circulating systems. Therefore, the mechanical principle of egg-laying is still obscure. Dr. Tchou-Su (1947-8) showed that the ovary could be induced to ovulate in vitro by hypotonic distilled water. He believed this was due to the absorption of water in hypotonic medium by the eggs which there-by swelled and burst the membrane. My results showed that the action of the pituitary gland seems but an addition of water.

The ovarian egg was found to absorb a large quantity of water and by the time of ovulation its water-content had increased by over one fifth of its total evaporated water wt. or by over one tenth of the total egg wet wt. This was so whether eggs were naturally or artificially ovulated.

Whether naturally ovulated, artificially ovulated, or artificially ovulated in vitro, the mechanical principle of ovulation is the same. The difference is that the abseorption of water is under the control of the pituitary gland in the first two, while it is caused by osmotic pressure in the last case.

SUMMARY

1. Injection of pituitary gland extract induced 21 frogs (*R. longicrus* & *B. melanostictus*) to ovulate before the normal breeding season. The effective number of glands needed in the case of *R. longicrus* (about 10) was more than in *B. melanostictus* (4-5).

2. Whether naturally or artificially ovulated, eggs before ovulation was found to have absorbed a large quantity of water. The water content continued to increase, and maturation enabled to occur. At the metaphase of the second maturation division the water content had gone up as high as 10% of the total egg wet weight.

I presume the increase of water content is the key to matuation and ovulation. This is the explanation of maturation from the physiological point of view.

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人工催產的蛙卵中所含水份的測計及其解釋

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生殖是一個大謎！目前的生物學，雖有相當進步，但仍只有觀察生殖現象的經過而細細測計其經過而已！在兩性生殖的高等動物裡，產卵可以說是生殖現象的開端。前人只知生殖期屆，卵巢中生長足够的卵球，勢必破巢而出，構成跌卵（Ovulation）或內產的現象。新近有些生物學家細研究產卵的原因，知道跌卵與腦下垂體前葉（鼻涕腺 Pituitary gland）有關。並且能用此腺中的霍爾蒙，注射到具卵的雌體中，促其早期產卵。這一類實驗的結果，人工不僅藉此明白產卵的眞因，而且已能控制產卵的動作！根據 Samartino 和 Rugh（1945），這一內分泌物對卵巢的作用是間接的，而且還須得神經系和循環器的幫助，才能有效。我師朱洗教授前曾證明：僅用蒸溜水的低壓，亦能使蛙類的卵巢在玻璃器中跌卵。這裡跌卵的原因是緣于卵在低壓環境中吸入外界的水份（$^1/_3$~$^1/_3$），因其膨脹的力量壓破卵巢膜，然後向外脫出。我們上面各表的統計數字，已經足够證明鼻涕腺的間接作用就是使卵加水。增加水份不僅是有關內產，而且還是促進成熟分裂的主要原因之一。不論是自然的產卵，不論是催促的產卵或玻璃器人爲的產卵，產卵的機械原理已知道是一樣的。所差的是前二者的吸水動作是受本體或異體的霍爾蒙的間接影響所引起，後者是受人工的純物理的滲透壓所引起而已。

提　　要

1. 用鼻涕腺（Pituitary gland）汁液的注射，可使臺灣產的兩種蛙類（*Rana longicrus*, *Bufo melanostictus*）提前產卵已無問題。惟 R. longicrus 所需的腺量（要十個）較 *B. melanostictus* 爲多（只需四、五個）。
2. 不論是自然的，或是人爲的，卵在離巢以前，必須先吸收外界的水份（約總量 3~4 %）作爲增新陳代謝之張本。續後水份繼續增進，成熟的分裂便接著發生。到成熟後期（二次成熟期分裂中期）時，卵得須增加水份10%左右。這水份的增進，我們認爲是發生內產或成熟現象的重要關鍵！